

Discovery of a Small Molecule Targeting *IRA2* Deletion in Budding Yeast and Neurofibromin Loss in Malignant Peripheral Nerve Sheath Tumor Cells

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Abstract

Malignant peripheral nerve sheath tumor (MPNST) is a life-threatening complication of neurofibromatosis type 1 (NF1). NF1 is caused by mutation in the gene encoding neurofibromin, a negative regulator of Ras signaling. There are no effective pharmacologic therapies for MPNST. To identify new therapeutic approaches targeting this dangerous malignancy, we developed assays in *NF1*^{+/+} and *NF1*^{-/-} MPNST cell lines and in budding yeast lacking the *NF1* homologue *IRA2* (*ira2Δ*). Here, we describe UC1, a small molecule that targets *NF1*^{-/-} cell lines and *ira2Δ* budding yeast. By using yeast genetics, we identified *NAB3* as a high-copy suppressor of UC1 sensitivity. *NAB3* encodes an RNA binding protein that associates with the C-terminal domain of RNA Pol II and plays a role in the termination of nonpolyadenylated RNA transcripts. Strains with deletion of *IRA2* are sensitive to genetic inactivation of *NAB3*, suggesting an interaction between Ras signaling and Nab3-dependent transcript termination. This work identifies a lead compound and a possible target pathway for NF1-associated MPNST, and shows a novel model system approach to identify and validate target pathways for cancer cells in which *NF1* loss drives tumor formation. *Mol Cancer Ther*; 10(9); 1740–50. ©2011 AACR.

Introduction

Neurofibromatosis type 1 (NF1) is a common genetic disorder in humans, occurring in 1 in 3,500 live births. NF1 is caused by inherited or *de novo* mutation in the *NF1* tumor suppressor gene, which encodes a GTPase activating protein (GAP) for Ras signaling proteins. NF1 has a broad clinical spectrum: affected individuals can develop benign nervous system tumors called neurofibromas, low-grade astrocytomas, pheochromocytoma, and juvenile myelomonocytic leukemia (1). Plexiform neurofibromas occurring in deep nerves can degenerate into malignant peripheral nerve sheath tumors

(MPNST), a life-threatening consequence of NF1 (2, 3). The lifetime risk of MPNST in NF1 patients is estimated to be 8% to 15%, and the 5-year survival is approximately 20% (4–6).

Plexiform neurofibromas are heterogeneous, consisting of fibroblasts, perineurial cells, mast cells, and Schwann cells, but only Schwann cells have biallelic inactivation of *NF1* (7). In mouse models, targeted deletion of *NF1* from the Schwann cell lineage gives rise to neurofibromas (8–10). Thus, loss of *NF1* from Schwann cell precursors is thought to initiate plexiform neurofibroma. Aberrant signaling occurs between *NF1*-deficient Schwann cells and *NF1* heterozygous mast cells, which generates a tumorigenic microenvironment (8, 11, 12). Because of their role in the initiation of plexiform neurofibroma and progression to MPNST, *NF1*-deficient Schwann cells represent an ideal population for targeted molecular therapies.

Chemical screens have revolutionized the discovery process for targeted molecular therapies. However, primary Schwann cells are difficult to culture and present a challenge for high-throughput screening. Another challenge in drug discovery is the rapid and efficient identification of the receptor for a novel compound—either the physical ligand or the biological process that is being modified. Approaches addressing these challenges are needed to identify new compounds and target pathways for the devastating tumors that afflict NF1 patients.

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Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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doi: 10.1158/1535-7163.MCT-11-0309

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The budding yeast *Saccharomyces cerevisiae* has 2 *NF1* homologues, *IRA1* and *IRA2*, which encode Ras-GAPs (13). Deletion of an *IRA* gene increases Ras-GTP and activates 2 pathways, a mitogen-activated protein kinase pathway that modifies cell morphology and the cyclic AMP dependent protein kinase (PKA) pathway (14, 15). Schwann cells lacking *NF1* have increased intracellular cyclic AMP and display PKA-dependent phenotypes (16, 17). The fact that Schwann cells lacking *NF1* and budding yeast lacking *IRA2* share the high PKA phenotype suggests that the yeast model might be useful for targeting the cell-autonomous effects of *NF1* loss in Schwann cells. The yeast platform enables rapid and cost effective high-throughput chemical screening and allows for the use of powerful yeast genetics to identify new drug targets.

To identify therapeutic agents and target pathways for NF1-associated tumors, we carried out a high-throughput chemical screen in mammalian MPNST cell lines and in the yeast. Here, we describe a novel compound that preferentially inhibits both a *NF1*-deficient MPNST cell line and *IRA2*-deficient yeast. In yeast, growth inhibition is partially alleviated by high-copy expression of *NAB3*, which encodes an RNA-binding protein that regulates transcript termination of nonpolyadenylated Pol II transcripts (18). This finding led us to a novel genetic interaction between *IRA2* and *NAB3*, suggesting a functional interaction between Ras signaling and the non-poly(A)-dependent termination pathway, which may have relevance to mammalian cells and the treatment of MPNST.

Materials and Methods

Yeast strain generation and high-copy suppressor screening

Strains and plasmids are listed in Table 1. *erg6Δ ira2Δ* were generated using standard 1-step PCR-based gene deletion methods (19, 20). Strains and plasmids used for high-copy suppressor screening are described in the Supplementary Fig. S2 legend. The YEp13-*NAB3* plasmid was generated from a suppressing YEp13 plasmid containing genomic DNA of chromosome XVI from 183393 to 188100 by excising a NcoI-XbaI fragment and re-ligating the construct. *nab3Δ nab3-3* was obtained from Dr. M. Swanson (University of Florida, Gainesville, Florida), and *nab3-11* was a gift from Dr. J. Corden (Johns Hopkins University, Baltimore, Maryland). *nab3-ts* alleles were confirmed by sequencing and introduced to the Σ 1278b genetic background by backcrossing. The *ctk1Δ::KANMX* allele was backcrossed to the Σ 1278b background from the Open Biosystems yeast deletion collection. Strains were backcrossed at least 4 times.

High-throughput screening

Screening was carried out at the University of Cincinnati Drug Discovery Center (DDC) using standard screening methodology. Full details of the screening

protocol are included in the Supplementary Materials and Methods.

Tissue culture

STS26T and T265 cells were routinely maintained in Dulbecco's Modified Eagle's Medium high glucose medium (Invitrogen 11965-092) with 10% FBS (Invitrogen 26140-079) and 1% penicillin/streptomycin (Invitrogen 15070-063). The cell lines and their original sources have been described (21). Each has been certified mycoplasma free and tested negative for a panel of mouse viruses. Their identity was validated by short tandem repeat genotyping. These cells had not previously been genotyped by this method, so there are no existing reference data. None of the genotypes match other cell lines in publicly accessible databases that have been queried. The cell lines are retested annually to ensure their ongoing identity. For drug sensitivity assays, 1,000 cells were plated to 96-well plates in 100 μ L medium. Plates were incubated for 1 day, then an additional 100 μ L of medium containing 0.2% dimethyl sulfoxide (DMSO) and twice the desired final concentration of UC1, a small molecule that targets 12 *NF1*^{-/-} cell lines and *ira2Δ* budding yeast, was added to the wells (final 200 μ L and 0.1% DMSO). Cells were incubated for 3 days, and viability was assessed by MTS assay. Three wells were analyzed for each condition, and the experiment was carried out 3 times.

Yeast drop assays

UC1 was added to 5 mL, 55°C molten synthetic complete agar from DMSO working solutions, mixed, and poured to tissue culture plates (Falcon 35-3002). Plates were uncovered for 20 minutes in a fume hood. Plates were prepared immediately before use. Log phase cultures were diluted to OD₆₀₀ = 0.08, 10-fold serial dilutions were carried out in Eppendorf tubes, and 5 μ L of each dilution was placed onto agar. Plates were incubated at 30°C or at the indicated temperatures for 3 days.

Yeast 96-well plate assays

Log phase cultures were diluted to OD₆₀₀ = 0.05 in synthetic complete medium, and 148.5 μ L cell suspension was added to 96-well plates containing 1.5 μ L DMSO or 1.5 μ L 100 \times compound (1% DMSO final). Plates were gently agitated, then incubated for 18 hours at 30°C in a humidified chamber. Optical densities were measured on a Molecular Devices Thermomax plate reader.

Yeast RNA extraction and reverse transcriptase PCR

Triplicate log phase cultures were treated with DMSO or UC1 for 8 hours at 30°C in 125-mL flasks. RNA was extracted from 5 to 10 \times 10⁶ cells with TriReagent (MRC TR-118) per manufacturer's instruction. Full details of the extraction method are listed in Supplementary Materials and Methods.

RNA was treated with DNase I (Roche 04716728001) according to manufacturer's instructions. DNase-I-treated RNA (200 ng) was used in a reverse transcription using

Table 1. Yeast strains used in this study

Strain	Genotype	Source
MLY41a	<i>ura3-52</i>	Lorenz (49)
MDW057	As MLY41a <i>erg6Δ::URA3</i>	This study
MDW035	As MLY41a <i>erg6Δ::URA3 ira2Δ::URA3-FOA^R</i>	This study
MDW230	<i>erg6Δ::HIS3 ira2Δ::URA3-FOA^R leu2-3 his3-11 ura3-52</i> [pMW001]	This study
MDW270	As MDW230, [YEp13] 5-FOA ^R	This study
MDW528	As MDW230, [pMW050] 5-FOA ^R	This study
YSW550-1D	<i>leu2Δ2 ura3-52 his3 trp1-289 nab3Δ2::LEU2 TRP1::nab3-3 α</i>	M. Swanson, University of Florida
YPN103	<i>ade2 can1-100 his3-11,15 trp1-1 leu2-3,112 ura3-1 nab3-11</i>	J. Corden, Johns Hopkins University
MDW547	<i>nab3Δ::LEU2 TRP1::nab3-3</i>	Cross YSW550-1D to MLY 4 times
MDW548	<i>ira2Δ::URA3 nab3Δ::LEU2 TRP1::nab3-3</i>	Cross YPN101 to MLY 4 times
MDW551	<i>nab3-11</i>	This study
MDW552	<i>ira2Δ::URA3 nab3-11</i>	This study
MDW561	<i>erg6Δ::URA3 nab3Δ::LEU2 nab3-3</i>	
MDW578	<i>ctk1Δ::KANMX erg6Δ::URA3</i>	
Plasmid	Description	Source
pMW001	pRS416- <i>ERG6</i>	This study
pMW050	YEp13-NAB3	This study

NOTE: Strains were generated as described in Supplementary Materials and Methods. All strains are mating type a, unless indicated.

Invitrogen SuperScript III Reverse Transcriptase (Invitrogen 18080-044) according to manufacturer's instructions. Reverse transcriptase (RT) reaction (2 μL) was used in a traditional PCR by using primers described in ref. 22. Cycling was carried out with 94°C denaturation, 50°C annealing, and 72°C extension for 0:45. For *PYK1* reactions, samples were removed after 22 cycles. For *SNR* readthrough reactions, samples were removed after 30 cycles.

Results

Identification of UC1 in chemical screens

To identify molecules with selective toxicity toward *NF1*-deficient cells, a chemical screen was carried out in MPNST lines with functional *NF1* (STS26T, *NF1*^{+/+}) and loss of *NF1* (T265, *NF1*^{-/-}; refs. 23, 24). We screened 6,000 compounds selected for chemical diversity from 250,000 compounds available through the University of Cincinnati DDC. The DDC library is chemically diverse and broadly represents the molecular space of drug-like compounds. The library is designed to remove nondrug-like molecules with unfavorable characteristics such as high reactivity, chemical instability, low solubility, and poor Lipinski profiles, thus maximizing the probability of finding a biologically relevant screening hit. Compounds showing greater inhibition against T265 than STS26T at 10 μmol/L were selected for follow-up. Hits were confirmed in triplicate and tested in a 10-point dose-response assay to define AC₅₀ values in both lines.

In parallel, compounds were screened in congenic budding yeast strains, 1 with deletion of *IRA2* (*ira2Δ*)

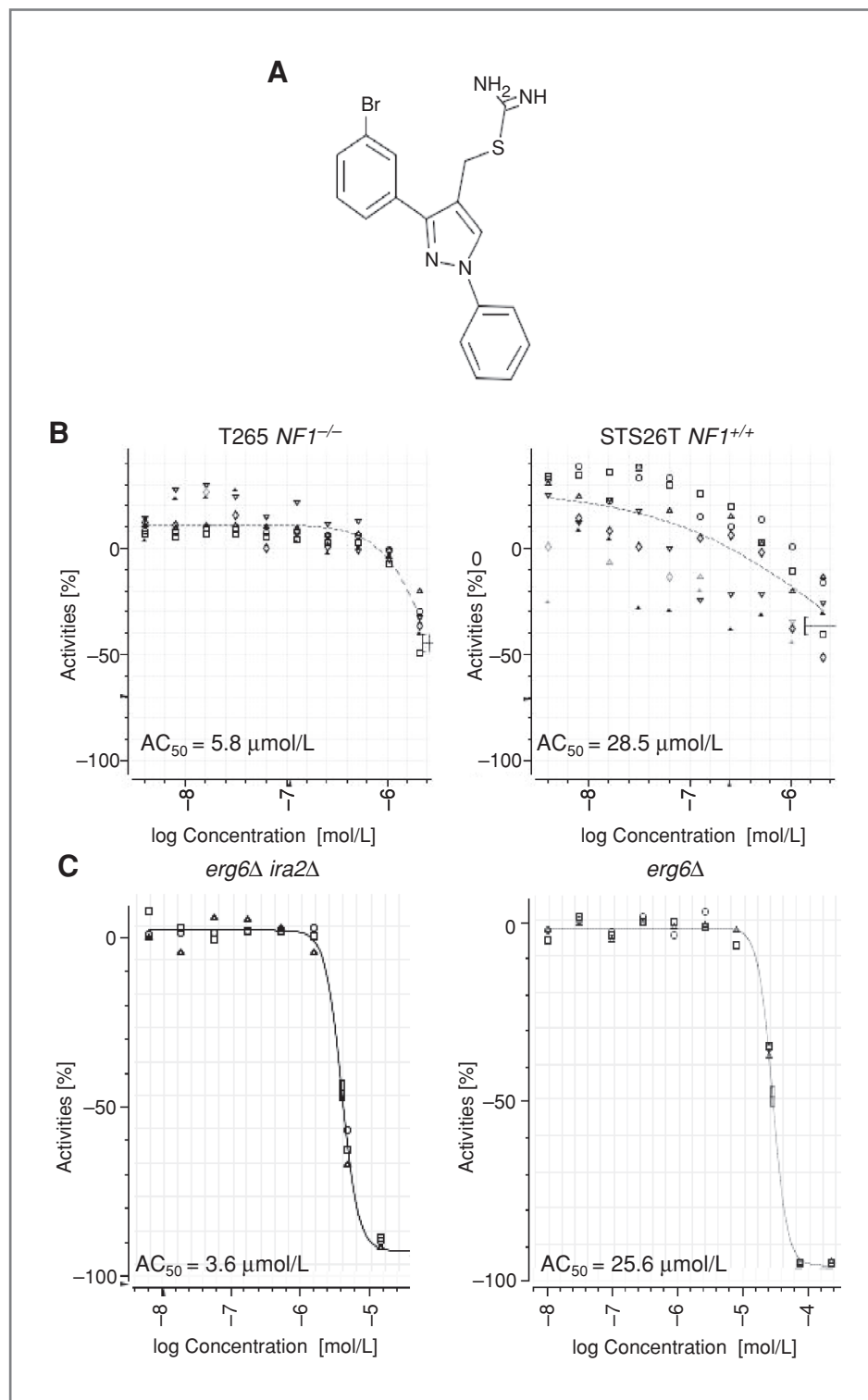
and 1 with an intact (wild-type) *IRA2*. Both strains had deletion of *ERG6* (*erg6Δ*), which increases cell permeability (25–29). We confirmed *ira2Δ* phenotypes including sensitivity to oxidative stress and failure to accumulate glycogen. Compounds were tested in both strains at approximately 20 μmol/L and confirmed hits having a greater inhibitory effect on *erg6Δ ira2Δ* than on *erg6Δ* were tested in a dose-response assay to define AC₅₀ values. We identified a set of compounds that inhibited both the T265 cell line and the *ira2Δ* budding yeast.

One compound, [3-(3-bromophenyl)-1-phenylpyrazol-4-yl]methyl carbamimidothioate hydrochloride, which we have named UC1 (Fig. 1A), was selected for follow-up based on its promising activity and commercial availability. In the cell line screen, UC1 had a greater activity against *NF1*^{-/-} T265 cells than the *NF1*^{+/+} STS26T cells (Fig. 1B). T265 cells were inhibited with an AC₅₀ of 5.8 μmol/L, whereas STS26T cells were inhibited with an AC₅₀ of 28.5 μmol/L. In the yeast screen, UC1 inhibited an *erg6Δ ira2Δ* strain at an AC₅₀ of 3.6 μmol/L, whereas the control *erg6Δ IRA2* strain was inhibited at 25.6 μmol/L (Fig. 1C). An identical compound lacking the metabromine showed reduced activity in *erg6Δ ira2Δ* cells, with an AC₅₀ value of 42.2 μmol/L ± 1.6 μmol/L (mean ± SD; Supplementary Fig. S1). This suggests that the metabromine is essential for the UC1 compound activity and shows that the yeast platform can be used to evaluate structure-activity relationships.

Confirmation of UC1 activity

UC1 activity was confirmed in the laboratory by using compound from an outside supplier (ChemBridge) to

Figure 1. High-throughput screening results for UC1. A, chemical structure of the UC1 molecule. B, UC1 activity in high-throughput screening of MPNST cell lines with differing *NF1* status. Dose-response curves for T265 (left) and STS26T (right) are shown. The x-axis represents the log of the UC1 concentration, whereas the y-axis represents the percent growth relative to control. C, dose-response curves for UC1 in budding yeast high-throughput screen. Dose-response curves are shown for *erg6Δ ira2Δ* (left) and *erg6Δ* (right), with x and y axes as described in B.



exclude possible effects of prolonged storage on the compound. UC1 decreased the number of viable T265 cells by the MTS assay with an AC_{50} of $3.35 \mu\text{mol/L}$ (95% CI: $2.41\text{--}4.29 \mu\text{mol/L}$; Fig. 2A). In contrast, STS26T had a significant fraction of viable cells even at the highest

dose tested ($10 \mu\text{mol/L}$), indicating a minimum 3-fold selectivity for the *NF1*^{-/-} cell line and confirming the differential sensitivity seen found in the screen. We tested MPNST-724, a non-NF1-associated MPNST cell line that retains neurofibromin protein expression (30, 31). This

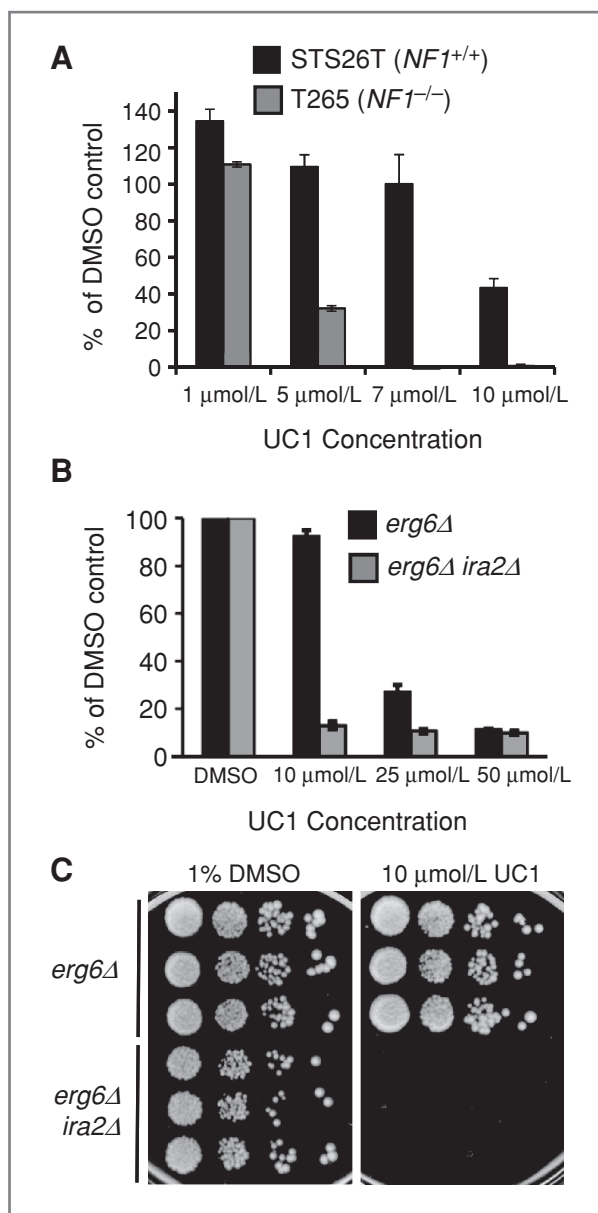


Figure 2. Confirmation of UC1 activity in yeast and cell lines. **A**, growth of STS26T and T265 cell lines in the presence of UC1. Values from MTS assay were normalized to control-treated samples and represent the mean \pm SD of 3 independent assays. **B**, a 96-well plate assay was carried out on *erg6Δ* and *erg6Δ ira2Δ* strains. Values are OD₆₀₀ normalized to DMSO-treated control wells and represent mean \pm SD of 3 wells per condition after 18 hours of growth. **C**, agar-based activity of UC1. A drop assay was carried out on agar containing UC1. Plates were incubated for 3 days at 30°C before photographing. Three independent cultures of each strain were tested.

line did not have dose-dependent growth inhibition up to 10 μmol/L UC1, suggesting that MPNST cells with functioning NF1 are not effectively targeted by UC1. To determine if UC1 was toxic toward non-MPNST cells, we tested a sarcoma cell line, HT1080, which was inhibited with an AC₅₀ of 9.02 μmol/L (data not shown).

In yeast, *IRA2* deletion in an *erg6Δ* strain increased sensitivity to UC1 in a 96-well plate growth assay (Fig. 2B), and 10 μmol/L UC1 completely inhibited the growth of an *erg6Δ ira2Δ* strain on agar medium (Fig. 2C). Increased permeability and loss of *IRA2* are necessary for UC1 activity, because *ERG6 IRA2* and *ERG6 ira2Δ* strains were not inhibited by UC1 (data not shown). These results confirmed the findings from the high-throughput screens and indicated that UC1 preferentially targets *NF1* and *IRA2* deficient cells.

Increasing levels of Nab3 suppresses UC1 sensitivity

We used a high-copy suppressor approach in yeast to identify potential targets for UC1. A UC1-sensitive strain was transformed with a high-copy plasmid library bearing fragments of the yeast genome and resistant colonies were isolated. High-copy suppressor screening required high-efficiency transformation of drug-sensitive cells. Although *erg6Δ* strains have been used in high-throughput screens, their utility in target identification studies is limited because of low transformation efficiency (29). To circumvent this issue, a plasmid shuffle approach was used (Supplementary Fig. S2). An *erg6Δ ira2Δ* strain bearing a rescue plasmid encoding *ERG6* was transformed with a high-copy library. The rescue plasmid was then counter selected, and 25,000 library transformants were plated onto 10 μmol/L UC1 agar medium. Resistant colonies were selected for follow-up. To identify constructs that were most informative for the UC1 mechanism, phenotypic assays were used to eliminate nonspecific suppressors—cells that regained the *ERG6* gene, restored PKA regulation, or were cross-resistant to a structurally unrelated compound (Supplementary Fig. S3). Plasmid DNA was isolated, shuttled, and unique constructs were identified by restriction digest. The genome regions and associated reading frames identified by DNA sequencing are shown in Table 2.

Suppressor constructs were retransformed into the parental strain for confirmation of UC1 resistance and sensitivity to the structurally unrelated compound. The most specific suppressor construct contained a region of yeast chromosome XVI containing 2 complete open reading frames, *NAB3* and *YPL191C*. Subcloning revealed that *NAB3* was responsible for UC1 sensitivity suppression. The resistance conferred by *NAB3* overexpression was easily appreciated on agar (Fig. 3A). *NAB3* encodes a single-stranded RNA binding protein that interacts with partners Nrd1 and Sen1, forming a complex that associates with the RNA Pol II C-terminal domain (CTD; 18, 32, 33). The complex directs termination and processing of small nuclear and nucleolar RNA transcripts (snRNA/snoRNA) and termination and degradation of antisense, intergenic, and select mRNA (34–36).

IRA2 and *NAB3* interact genetically

One explanation for *NAB3* overexpression suppressing UC1 sensitivity is that UC1 is targeting Nab3 and

Table 2. High-copy suppressors of UC1 sensitivity

No resistance to unrelated compound				
Chromosome	Start	End	# Isolated	Full-length ORFs
XV	1032412	1037214	1	<i>NDD1</i>
XII	1000673	1007148	1	<i>IMD3, CNA1, TSR2</i>
XIII	350140	355586	1	<i>YET2, ARA2, ARG80, MCM1</i>
XVI	183393	188100	1	<i>NAB3, YPL191C</i>
Slightly resistant to unrelated compound				
Chromosome	Start	End	# Isolated	Full-length ORFs
XIII	913156	Ty element	1	Unknown
XV	380485	389109	2	<i>BUB3, STI1, CIN5, DFG16</i>
XV	1032412	1037214	3	<i>NDD1</i>
VII	954571	961868	4	<i>YHB1, PHO81, NAS6</i>
XIII	423056	433459	2	<i>SEC14, NAM7, ISF1</i>
XV	836614	849743	1	<i>HEM4</i>
VII	957743	961496	1	<i>YHB1</i>
IX	166131	175721	1	<i>MOB1, SLM1, SHQ1, DPH1, XBP1</i>
XV	1032413	1037214	1	<i>NDD1</i>
VII	260828	265887	1	<i>ITC1, SNT2</i>
Highly resistant to unrelated compound				
Chromosome	Start	End	# Isolated	Full-length ORFs
XIII	250991	256880	1	<i>MRPL39, ERG6, YAP1, GIS4</i>
XVI	24342	Ty element	3	Unknown
XIII	556382	561831	7	<i>TIF34</i>
XV	840150	845510	3	<i>HEM4, CAF20, RFM1</i>
XIII	251832	256880	2	<i>ERG6, YAP1</i>
XIII	555224	561831	2	<i>TIF34</i>

NOTE: Constructs were isolated from UC1-resistant colonies as described in the text and in Materials and Methods. Constructs were identified by DNA sequencing.

Abbreviation: ORF, open reading frame.

compromising its function. In this model, *NAB3* overexpression confers resistance by increasing the quantity of Nab3 protein, so that higher concentrations of UC1 are required for inactivation. This predicted that deletion of *NAB3* would be synthetically lethal with *IRA2* deletion. At the time of our studies, no such interaction had been reported. *NAB3* is essential, so temperature sensitive (*ts*) alleles of *NAB3* were used to test for a genetic interaction. A synthetic lethal effect between *ira2Δ* and a *nab3-ts* strain is indicated by lack of growth of *ira2Δ nab3-ts* double mutants at a temperature that permits growth of the single mutants. Two *nab3-ts* alleles were introduced to Σ 1278b strains wild-type or null for *IRA2* (Fig. 3B, adapted from ref. 33). Both alleles showed a negative genetic interaction with *ira2Δ*. Strains with a *nab3-11* allele grew at 27°C and failed at 30°C, whereas *ira2Δ nab3-11* strains grew poorly at 27°C (Fig. 3C). Similar results were obtained with *nab3-3*. Although *nab3-3* strains grew at 34°C, *ira2Δ nab3-3* strains grew poorly at this temperature (Fig. 3D). This evidence showed that an *ira2Δ* strain is more sensitive to Nab3 dysfunction than an *IRA2* strain. Our discovery is consistent with a recent report of genetic interactions between the Ras-PKA pathway and Nab3-Nrd1

(M. M. Darby, X. Pan, L. Serebreni, J. D. Boeke, and J. L. Corden, submitted for publication).

UC1 does not inhibit the role of Nab3 in transcript termination

A genetic interaction supported the possibility of Nab3 as a target in *IRA2*-deficient yeast. To determine if UC1 was acting directly to inhibit Nab3, a readthrough transcription assay was carried out. Inactivation of Nab3 or Nrd1 generates extended transcription products downstream of snoRNAs which can be detected by reverse transcriptase (RT)-PCR with primers depicted in Fig. 4A (adapted from ref. 22). We validated this detection approach by using the *nab3-ts* strain generated from earlier experiments (data not shown, Fig. 5). *erg6Δ* and *erg6Δ ira2Δ* cultures were treated with vehicle or 10 μmol/L UC1 for 8 hours and analyzed for readthrough transcription by RT-PCR. This treatment inhibited the growth of *erg6Δ ira2Δ* by 86% and that of *erg6Δ* strain by 62% (Fig. 4B). Despite inhibition in both strains, no increase in readthrough transcription at either of 2 snoRNA loci were detected (Fig. 4C).

Dysfunctional Nab3-Nrd1 leads to markedly increased *NRD1* mRNA levels by alleviating negative feedback on

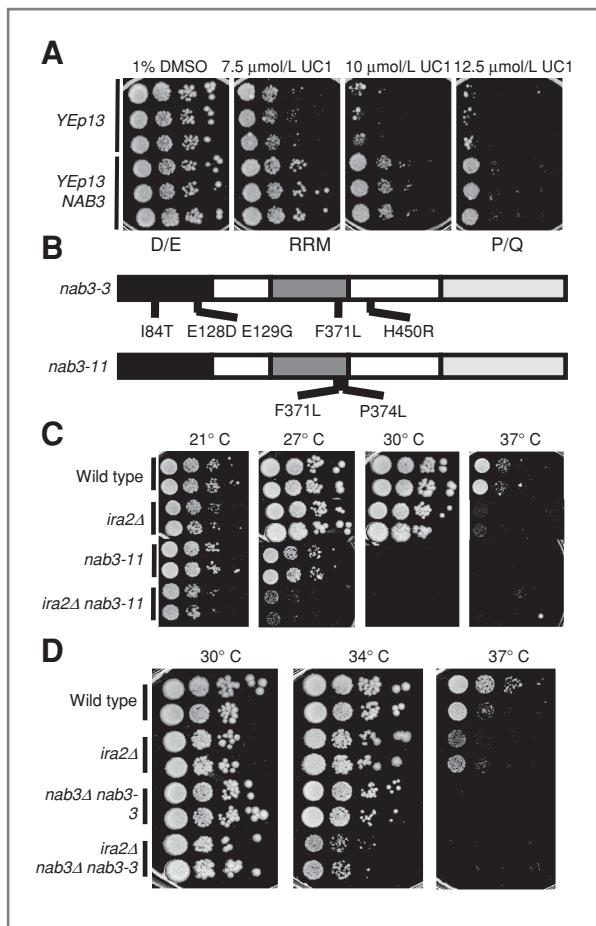


Figure 3. *NAB3* suppresses UC1 sensitivity and interacts genetically with *ira2Δ*. A, a drop assay on synthetic complete medium was carried out with increasing doses of UC1 on 3 independent cultures of *erg6Δ ira2Δ* containing the empty vector *YEp13* or the vector plus a fragment containing *NAB3*. Plates were incubated for 3 days at 30°C before photographing. B, *nab3-ts* alleles analyzed with *IRA2* deletion. D/E represents an N-terminal acidic domain, RRM represents the RNA recognition motif, and P/Q represents a C-terminal proline/glutamine rich domain. C and D, $\Sigma 1278b$ background strains were analyzed in drop assays at the indicated temperatures after 3 days of growth on synthetic complete medium. Two strains of each genotype were analyzed.

NRD1 expression, providing a sensitive assay for detecting Nab3-Nrd1 complex activity (34). We did not detect significant increases in *NRD1* mRNA levels in samples from UC1-treated cultures by real-time RT-PCR (data not shown). Along with the lack of readthrough transcription, this result indicated that UC1 is not likely to have a direct inhibitory effect on Nab3-Nrd1-dependent termination.

Functional homologues of Nab3 and Nrd1 have not yet been identified in mammals. However, Nrd1 bears structural resemblance to the SCAF4 and SCAF8 C-terminal-associated splicing factors, whereas Nab3 is similar to HNRNPC, an RNA binding protein. HNRNPC was identified in an siRNA screen to identify targets in cells with activating point mutations in Ras,

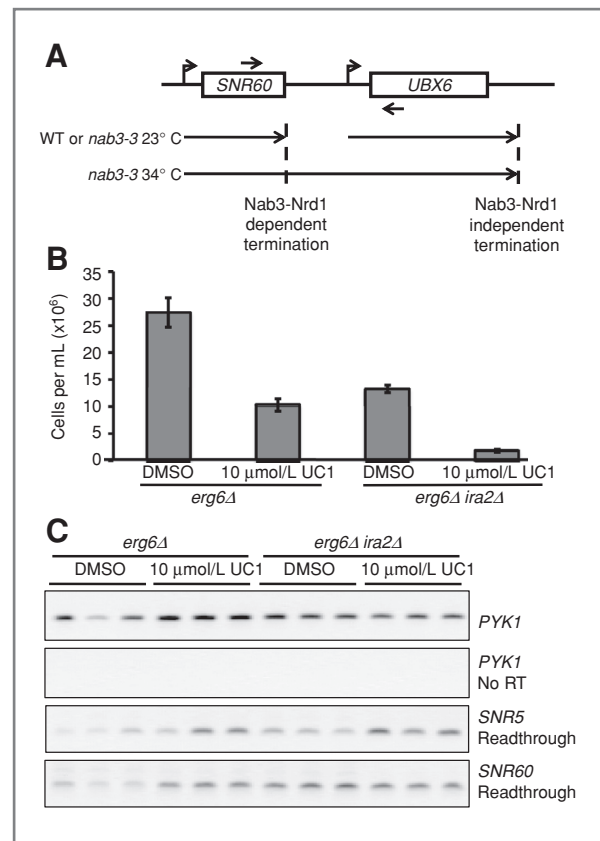


Figure 4. Nab3 inactivation confers UC1 sensitivity. A, schematic of readthrough detection. See text for details. B, triplicate cultures of *erg6Δ* and *erg6Δ ira2Δ* were treated with DMSO or UC1 for 8 hours. Strain growth was monitored by OD₆₀₀ and converted to cells per milliliter. Values are the mean \pm SD of 3 cultures. C, RT-PCR detection of readthrough transcripts in *nab3-3* and UC1-treated samples. Samples from cells treated in B were processed for RNA extraction and RT-PCR as described in Materials and Methods. The presence of product indicates readthrough transcription at the *SNR* locus.

suggesting that HNRNPC may be a target in Ras-deregulated cells (37). In preliminary assays, knockdown of HNRNPC or SCAF8 by short hairpin RNA inhibited STS26T and T265 cell lines equally (data not shown). Because knockdown of these factors was not selectively toxic toward the *NF1*-deficient cell line, we concluded that neither factor is likely to be the molecular target of UC1 in mammalian cells.

Nab3 inactivation confers UC1 sensitivity

Our data established that UC1 sensitivity is suppressed by overexpression of *NAB3*. However, assays in *erg6Δ* and *erg6Δ ira2Δ* cells showed that UC1 did not disrupt Nab3-dependent termination. We considered that UC1 sensitivity could result from Ras activity downregulating Nab3. This would explain the *ira2Δ/nab3-ts* genetic interaction; if high Ras activity because of *ira2Δ* compromised an essential Nab3 activity, cells would be more sensitive to further disruption of a specific activity of Nab3 by UC1. This model predicted that drug permeable *IRA2* cells

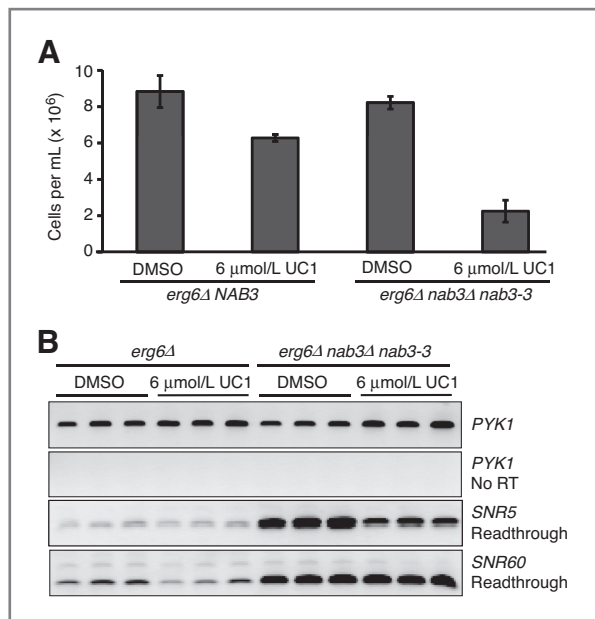


Figure 5. UC1 inhibits cell growth without increasing readthrough. **A**, growth of *erg6Δ* and *erg6Δ nab3-3* strains treated with UC1 for 8 hours. Cell density in triplicate cultures was determined by OD₆₀₀ as in Fig. 4. **B**, RT-PCR for SNR termination defects were carried out as described in Materials and Methods and in Fig. 4.

with Nab3 dysfunction would be UC1 sensitive. Therefore, we tested the UC1 sensitivity of an *erg6Δ nab3-3* strain at a semi-permissive temperature. Under these conditions, 6 μmol/L UC1 inhibited the *erg6Δ* control strain by 28% after 8 hours, whereas the *erg6Δ nab3-3* strain was inhibited by 73% (Fig. 5A). The *nab3-ts* strain showed noticeably increased readthrough transcription. Consistent with our previous data, UC1 treatment did not increase readthrough in either strain (Fig. 5B). This indicated that downregulation of Nab3 activity leads to increased UC1 sensitivity in the setting of normal Ras signaling. *IRA2* deletion did not lead to readthrough transcription (Fig. 4C, compare DMSO samples for *erg6Δ* and *erg6Δ ira2Δ*), indicating that Ras activation may be modifying a specific function of Nab3, rather than globally deregulating Nab3-dependent termination.

ctk1Δ* confers UC1 sensitivity and interacts genetically with *ira2Δ

The Nab3-Nrd1 complex acts in association with the CTD of RNA Pol-II. This association is partly regulated by the yeast CTD kinase Ctk1, which phosphorylates the CTD and opposes the association of Nab3-Nrd1 with the Pol II holoenzyme (33, 38, 39). *ctk1Δ* rescues the temperature sensitivity of an *nrd1-ts* allele by altering the CTD phosphorylation state and promoting Nab3-Nrd1 association (33). We questioned whether UC1 could be interfering with Nab3 recruitment to the CTD, which could compromise other functions of Nab3 in addition to non-poly(A) transcript termination. This model predicted that

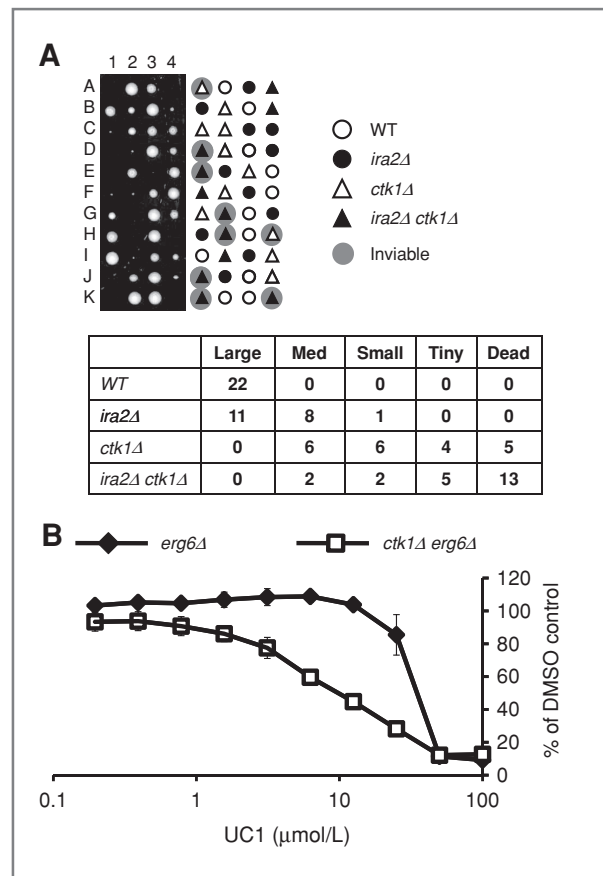


Figure 6. *ctk1Δ* interacts genetically with *ira2Δ* and sensitizes *erg6Δ* cells to UC1. **A**, a diploid *ira2Δ::URA3/IRA2 ctk1Δ::KANMX/CTK1* was sporulated and dissected to yeast peptone dextrose agar. Plates were grown for 3 days at 30°C. Colony size was scored, then genotypes were determined by stamping to C-Ura and G418 medium. Because of genetic markers, *ira2Δ* colonies grow on C-Ura whereas *ctk1Δ* colonies grow on G418 allowing full identification of the genotype for each colony. **B**, three cultures of *erg6Δ* and *erg6Δ ira2Δ* were treated with UC1 in a 96-well plate dose-response assay. Growth after 18 hours of incubation is expressed as percentage of DMSO control OD₅₉₅. Values are mean ± SD of 3 cultures.

CTK1 deletion would rescue UC1 sensitivity by promoting the Nab3-CTD interaction. In generating strains to test this model, we observed synthetic lethality between *ctk1Δ* and *ira2Δ*. In tetrad analysis, *ctk1Δ* spores were viable, whereas the double mutant *ctk1Δ ira2Δ* spores formed minute colonies (Fig. 6A). This result was unsurprising, as *CTK1* deletion as well as other mutations that interfere with the Pol II CTD are synthetically lethal with Ras activation (40–42).

Because of the *ira2Δ/ctk1Δ* negative genetic interaction, we tested for a *ctk1Δ/UC1* interaction in an *erg6Δ IRA2* background. In contrast to our original prediction, an *erg6Δ ctk1Δ* strain was more sensitive to UC1 than *erg6Δ* (Fig. 6B). This unexpected result indicated that *CTK1* deletion is sufficient to increase UC1 sensitivity. Thus, 3 different genetic modifications that are linked to Pol II CTD function—*ira2Δ*, *ctk1Δ*, and *nab3-ts*—all conferred

UC1 sensitivity, suggesting that UC1 is impinging on Pol II activity. The mechanism of impingement, including the receptor for UC1 and whether this is a conserved target, is an avenue of future investigation.

Discussion

This work illustrates the power of integrating mammalian and model systems to identify new compounds and explore therapeutic targets for cancer treatment. We used human cell line and budding yeast chemical screens to identify candidate compounds for the treatment of tumors associated with NF1. While this work was underway, the MPNST cell lines that we used in our study were used to characterize other small molecules targeting *NF1* loss (43). We identified UC1, a compound targeting *NF1*-deficient mammalian cells and budding yeast lacking the *NF1* homologue *IRA2*. We used yeast genetic approaches to identify a genetic suppressor of UC1 sensitivity. This led to the discovery of a genetic interaction between the Ras signaling pathway and the machinery that regulates transcript termination, a finding that has been confirmed by independent studies (M. M. Darby, X. Pan, L. Serebreni, J. D. Boeke, and J. L. Corden, submitted for publication). This approach identified a candidate small molecule for pharmacologic development, as well as a potential novel target pathway for treating NF1-associated tumors.

Identification of UC1 and cross-species validation

Although differential sensitivity of UC1 in the MPNST cell lines may be caused by *NF1* loss, these cell lines are not isogenic and other genetic differences could be responsible for differential sensitivity. UC1 activity in *IRA2*-deficient budding yeast is significant because the yeast strains used—*erg6Δ IRA2* modeling an *NF1*^{+/+} cell and *erg6Δ ira2Δ* modeling an *NF1*^{-/-} cell—are derived from the same parental strain and are congeneric. Consequently, differential activity in budding yeast cells strongly suggests that UC1 is targeting the *ira2Δ* phenotype, a model of *NF1*^{-/-} status in mammalian cells.

NAB3 expression suppresses UC1 sensitivity

The yeast system can be used to identify the potential mechanism of action of new compounds. We identified *NAB3* as a high-copy suppressor of UC1 sensitivity and found that Ras deregulation renders cells more sensitive to Nab3 inactivation. Because *NAB3* is an essential gene, it is often not included in high-throughput genetic screens, which may explain why this interaction has not been uncovered previously (44). It was previously noted that mutations in the yeast adenylate cyclase *CYR1* rescue the temperature-sensitive defect of an *nrd1* temperature sensitive strain (33).

Evidence against direct inhibition of Nab3 by UC1

With Nab3 or Nrd1 inactivation, transcription termination at snRNA and snoRNA is compromised, resulting in

readthrough transcription (18). We did not find evidence for readthrough in UC1-treated cells, suggesting that UC1 does not cause a non-poly(A)-dependent termination defect. This might indicate that UC1 is not targeting Nab3. However, lethality in *NAB3* mutants might be because of deregulation of a subset of essential genes, and we cannot rule out the possibility that UC1 causes termination defects at specific loci. Alternatively, UC1 might inhibit an essential function of Nab3 that is unrelated to termination. Two research groups have implicated Nab3-Nrd1 in silencing Pol II transcription at the rRNA encoding DNA (rDNA) array (45, 46). UC1 might modify Nab3 activity at rDNA, but not at other termination sites in the genome.

Other potential mammalian homologues of Nab3-Nrd1

The Nab3-Nrd1 complex can limit expression of certain genes bearing Nab3-Nrd1 termination motifs in the 5'-coding region (34). Therefore, Nab3-Nrd1 limits Pol II activity in a gene-specific manner. In mammalian cells, the negative elongation factor (NELF) and DRB sensitivity inducing factor (DSIF) negatively regulate Pol II. These factors are opposed by P-TEFb, the mammalian homologue of yeast Ctk1. It has been proposed that the Nab3-Nrd1 complex in yeast plays a similar role in transcription as NELF/DSIF in mammals (33). Synthetic lethality between *IRA2* and *CTK1* deletion suggests that targeting Cdk9, the kinase component of pTEFb and the mammalian homologue of Ctk1, could be a therapeutic strategy for MPNST.

Conclusion

We identified 2 genetic interactions in yeast that suggest target pathways for NF1-associated malignancy; first, the functional homologue of Nab3 and second, the functional homologue of Ctk1. This work shows that yeast model systems can identify lead compounds for development in the treatment of human cancer. We have developed an approach for genetic suppressor screening in the *erg6Δ* background, which previously limited the application of yeast-based chemical screens. Genetic approaches in yeast can be used to identify potential target pathways for novel compounds. The cell lines used in this work are suitable for xenograft assays, allowing for future investigation of UC1 activity in an *in vivo* model (47, 48). This technology may be applied to synthetic lethality screening for other conserved tumor suppressor genes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank members of the Sanchez Laboratory for their comments; Dr. Craig Tomlinson and Heidi Trask of the Dartmouth Genomics and Microarray Facility; and Dr. Jeffrey Corden and Miranda Darby of Johns

Hopkins University for analysis of real-time RT-PCR data and for sharing unpublished results.

Grant Support

This work was supported by Prouty Norris Cotton Cancer Center Funds (Y. Sanchez) and NIH/NINDS R21NS060940 (Y. Sanchez and N. Ratner), NIH training grant T32-CA009658-18 (Y. Sanchez), and by a Children's Tumor

Foundation Young Investigator Award (M. Wood). M. Wood is an Albert J. Ryan fellow.

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Received April 25, 2011; revised June 13, 2011; accepted June 13, 2011; published OnlineFirst June 22, 2011.

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Discovery of a Small Molecule Targeting *IRA2* Deletion in Budding Yeast and Neurofibromin Loss in Malignant Peripheral Nerve Sheath Tumor Cells

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Mol Cancer Ther 2011;10:1740-1750. Published OnlineFirst June 22, 2011.

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